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Susceptibility of *Lactobacillus pentosus* strains isolated from fermented products to streptomycin and kanamycin

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Abstract

Streptomycin and kanamycin susceptibility of *Lactobacillus pentosus* P and Ind-3 were tested with paper discs in MH agar by disc diffusion techniques. The antibiotic susceptibility was evaluated according to the NCCLS standard. Genes responsible for resistances to streptomycin [aadA and aadE], and kanamycin [aph(3")-III] were detected by PCR. The sequences of the target band were detected and compared using the BLAST program. The results showed that *Lactobacillus pentosus* P and Ind-3 were resistant to kanamycin. To streptomycin, the strain Ind-3 was resistant, but P was sensitive. And the kanamycin-resistant gene was in plasmid. The resistant plasmid was highly similar to Enterococcus faecalis plasmid pSL1. Currently, new beneficial bacteria are being developed continuously into market. If they showed antibiotic resistance and the resistance can be transferred to other bacteria, especially pathogenic microorganisms, it will be a serious social problem. So, the antibiotic resistance should be checked firstly before subsequent utilization. The results confirm that antibiotic susceptibility was a very important feature in the selection of potentially probiotic lactic acid bacteria.

Introduction

Lactic acid bacteria (LAB) are a group of diverse species taxonomically which can convert fermentable carbohydrates into lactic acid. They often occupy a wide range of natural environments, including the gastrointestinal tract of humans and animals as well as foods found commonly in meat, milk and cheese. Due to their claimed functions for gut function and health, lactic acid bacteria are of particular interest in various fields. Traditionally, some lactic acid bacteria, like *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, have been used safely for a long history. They are agreed to be security and did not have the pathogenic possibility. Currently, new beneficial bacteria are being developed continuously into market. However, the security of these new strains has caused great concern, especially their antibiotic sensitivity that should be an important part of safety assessment (Huys *et al.*, 2002; Coppola *et al*., 2005; Patrice, 2006).

Studies have shown that some strains from *Lactobacillus*, *Bifidobacterium* and *Streptococcus* used in yogurt may carry resistance genes (Charteris *et al*., 1998a; Morten, 2002; Osman and Bulent, 2004; Labia *et al.*, 2008; Roberta *et al.*, 2010). Generally, the worry about resistance transfer was not necessary if antibiotics were not used widely. Actually, abuse of antibiotics has become a serious social problem, and created a selective pressure for acquisition of resistance phenotypes which can be transmitted via food carrier (Teuber *et al*., 1999). For instance, dissemination of some antibiotics through milk products, which are used against the breast inflammation of cows, has been an important public health issue (Teuber *et al.*, 1999). Clearly, the resistance of lactic acid bacteria to antibiotics which are employed as probiotics or dietary adjuncts should be checked before subsequent utilization.

The evolution of antibiotic-resistant and foodborne pathogens has been widely reported (Threlfall *et al.*, 2000; White *et al*., 2002; Walsh *et al*., 2008). However, in recent years, fermented food products have received increasing attention as potential vehicles of spread of antibiotic resistance determinants, which might be horizontally transferred to opportunistic pathogens within complex microbial communities such as the gut microflora. The resistance and resistance-related genes of *Bifidobacterium*, *Lactobacillus* and *Pediococcus* strains to different

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antibiotics was reported systematically (Huys *et al*., 2004; Hummel *et al.*, 2007; Maria *et al*., 2007). The tetM gene transfer of tetracycline resistance in *Lactobacillus plantarum* among strains was showed by Niamh *et al*. (2010).

L. pentosus is a special kind of bacteria. It is present naturally in raw milk. *L. pentosus* plays an important role during the ripening of cheese mostly as non-starter lactic acid bacteria. And it is used widely as a spontaneous starter in the dairy, vegetable and meat fermentation. The development of *L. pentosus* in milk provides optimal conditions for curd formation, prevents the outgrowth of pathogenic and spoilage bacteria, and creates biochemical conditions for ripening. Further, *L. pentosus* participates in the development of cheese texture and flavor via its proteolysis and amino acid catabolic systems. However, the antibiotic sensitivity, antibiotic gene and the gene transfer of *L. pentosus* were not yet been studied.

In our study, *L. pentosus* P and Ind-3, isolated respectively from fermented products manufactured in China were studied. Streptomycin and kanamycin are two of the most widely used antibiotics in both clinical and animal therapy. The aim of this study was to evaluate their susceptibility to these two antibiotics. The resistance-related gene was also investigated. This will be helpful to promote the safety evaluation and development of potentially probiotic lactic acid bacteria.

Materials and Methods

Bacterial strains and cultivations

L. pentosus strains P and Ind-3, isolated from fermented products and deposited now in China Industrial Culture Collection (CICC, Beijing), were included in the study. They were activated in skim milk medium and MRS broth. 1% of two strains were inoculated to MRS broth, and then cultivated at 37°C for 10 h before further use.

Testing for antibiotic susceptibility

Antibiotic susceptibility was semi-quantitatively determined with paper discs contained streptomycin and kanamycin by disc diffusion referring to the National Committee for Clinical Laboratory Standards (NCCLS) as described by Charteris *et al.*, (1998b). Briefly, 1.0 ml bacterial suspension tested (approximately 1.5×10^8 CFU/ml) was added to sterile petri dish with diameter of 90 mm, and then mixed with a 15 ml MH agar (Muller Hinton Agar: beef extract powder 6 g/L, casein hydrolysate 17.5 g/L, soluble starch, 1.5 g/L, agar 17 g/L, pH 7.3 ± 0.1) until the medium solidified. The sensitivity discs were pasted closely onto the solidified medium with sterile tweezers after 5 min at room temperature. Three discs were pasted in each dish. The distance was more than 24 mm of each disc center. The distance was more than 15 mm from disc edge to the inner edge of dish. Next, the dishes were placed at room temperature for 1.5 h and then incubated at 37°C. After 24 h, the inhibition zone measured around the antibiotic disc with vernier caliper was recorded. Three replicates of each antibiotic were done, and the results were averaged.

Standard sensitive strains of *Staphylococcus aureus* ATCC 25923 was used as quality control strain. The operation was same with the above. The antibiotic susceptibility of the tested strains was evaluated according to the standard disc diffusion method of NCCLS criteria (Table 1).

Extraction of plasmid DNA

Plasmid DNA of *L. pentosus* P and Ind-3 was extracted and purified following the procedure of Giorgio (2000).

Detection of resistance genes

With the plasmid template, genes responsible for resistances to streptomycin [aadA and aadE], and kanamycin [aph(3")-III] were detected by PCR. The primers described in Table 2 were synthesized by Beijing Genomics Institute. The reaction concentrations and thermal cycling programmes were performed as conferences in Table 2. PCR amplicons were separated by conventional 0.7% (w/v) agarose gel electrophoresis (100 V, 4°C) in TAE buffer and visualized by ethidium bromide staining.

The target bands were recovered. The sequences were detected by Beijing Genomics Institute, and then compared to others in public databases using the BLAST program.

Results

Antibiotic susceptibility

Kanamycin and streptomysin are aminoglycoside antibiotics, and therefore protein synthesis inhibitors. Some bacteria were susceptible to the two antibiotics. So, they were used widely in human and animals. The inhibition of kanamycin and streptomysin towards the two strains incubated for 24 h at 37°C was detected, and listed in Table 3. The antibiotic sensitivity of L. pentosus strains was determined according to Table 2 and was shown in Table 4. *L. pentosus* Ind-3 and P showed resistance to kanamycin. The tolerance of *L. pentosus* to streptomysin exhibited strain-specific.

Table 1. Antibiotic susceptibility test and criteria for inhibition zone diameter

Antibiotics	Content (μg/disc)	criterion for inhibition zone diameter (mm)		
		R	M	S
Streptomycin	10	≤11	12～14	≥15
Kanamycin	30	≤13	14～17	≥18

Note: S-susceptible; R-resistant; M-Moderately sensitive.

Table 2. Primers used for PCR detection of tetracycline and kanamycin resistance-related genes (Labia *et al.*, 2008)

Primer pair	Primer sequence(5′→3′)	Product size (bp)	PCR cycles and conditions
aph(3")-F	GCCGATGTGGATGCGAAAA	292	for 1 min, for 1 min, for 20 s; 30 cycles
aph(3")-R	GCTTGATCCCCAGTAAGTCA		
aadAF	ATCCTTCGGCGCGATTTTG	735	for 1 min, for 1 min, for 1 min; 30 cycles
aadAR	GCAGCGCAATGACATTCTTG		
aadEF	ATGGAATTATTCCCACCTGA	565	for 1 min, for 1 min, for 40 s; 30 cycles
aadER	TCAAAACCCCTATTAAAGCC		

Table 3. Inhibition zone of *L. pentosus* Ind-3 and P to kanamycin and streptomycin in MH agar medium (mm)

antibiotic	Inhibition zone diameter (mm)	
	P	Ind-3
kanamycin	10.0±0.2	12.5±0.3
streptomycin	18.2±0.3	0±0.2

The MH agar plates with antibiotics discs were incubated at 37°C for 24 h. The inhibition zone was measured (mm). Data are mean ± SE

Table 4. Antibiotic sensitivity of *L. pentosus* Ind-3 and P to kanamycin and streptomycin. The antibiotic susceptibility was determined according to Table 1.

antibiotic	antibiotic susceptibility	
	Strain P	strain Ind-3
kanamycin	R	R
streptomycin	S	R

note: R-resistant, M -Moderately sensitive, S-sensitive

Strain Ind-3 was resistant to streptomycin, but P was sensitive.

PCR studies

Akter *et al.* (2011) reported that the percentage of amoxicillin resistant bacteria in tomato and carrot samples in Dhaka city were 3.4% and the resistance to amoxicillin is plasmid mediated. Some studies also indicate that antibiotic resistance genes are generally carried on plasmids, and can be transferred to other bacteria by means of conjugation (Pier *et al.*, 2003). It can be concluded that a main threat associated with these lactic acid bacteria might be whether they contain potential resistant-plasmid, since highly antibiotic resistant pathogenic bacteria might be resulted in this case. So, it is important to determine whether antibiotic resistance genes are present on chromosomes or on plasmids.

In the study, the primers aadA and aadE were used to amplify the streptomycin resistance gene (Table 2). As shown in Figure 1, no PCR product was amplified in the streptomycin-resistant strain of *L. pentosus* Ind-3.

The primer aph(3")-III was used to amplify kanamycin resistance gene in *L. pentosus* strains P and Ind-3 (Table 2). As shown in Figure 2, one band about 292 bp was shown in PCR products of strains Ind-3 and P, respectively.

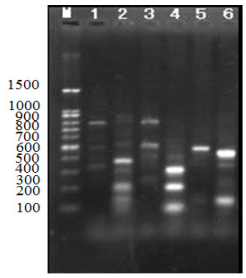

Figure 1. PCR analysis of aadA and aadE in *L. pentosus* Ind-3. With primers of aadA and aadE, the PCR amplifications were conducted with the plasmid template in strain Ind-3. The PCR amplicons were separated by 0.7% (w/v) agarose gel electrophoresis (100 V, 4°C). Lane M: reference standard marker (100 bp -1500 bp); Lane 5: aadA amplicon of strain Ind-3; Lane 6: aadE amplicon of strain Ind-3.

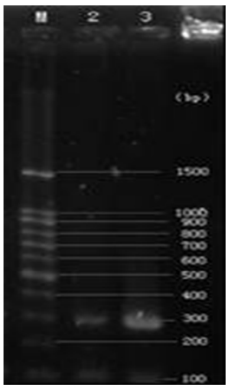

Figure 2. PCR analysis of aph (3'')-III in *L. pentosus* strains Ind-3 and P. With primer of aph(3")-III, the PCR amplifications were conducted with the plasmid template in strains Ind-3 and P. The PCR amplicons were separated by 0.7% (w/v) agarose gel electrophoresis (100V, 4°C). Lane M: reference standard marker (100 bp - 1500 bp); Lane 2: aph(3")-III amplicon of strain Ind-3; Lane 3: aph(3")-III amplicon of strain P.

Data from Figure 1 and Figure 2 show that the two tested strains have a plasmid which likely contains kanamycin resistance gene.

Sequencing and alignment

The fragment of 292-bp in *L. pentosus* strain P (as shown in Figure 2) was recovered and then was sequenced. The sequence is given as follows:

GGCGGACTCATTTAAGATCCGCGCGAGCTGTATGATTTTTTAAAGACGGAAAAGCCCGAAGAGGA
ACTTGTCTTTTCCCACGGCGACCTGGGAGACAGCAACATCTTTGTGAAAGATGGCAAAGTAAGTGGC
TTTATTGATCTTGGGAGAAGCGGCAGGGCGGACAAGTGGTATGACATTGCCTTCTGCGTCCGGTCGA
TCAGGGAGGATATCGGGGAAGAACAGTATGTCGAGCTATTTTTTGACTTACTGGTGATCAAGCAAA

This sequence alignment was done with NCBI blast, and found to have a high similarity to

Enterococcus faecalis plasmid pSL1 (21918 bp). The identity between these two bacteria is 97%. Our present study indicates that, to a large extent, the kanamycin resistance of *L. pentosus* strain P was determined by aph(3")-III gene in plasmid.

Discussion

In recent years, more researches have been done on antibiotic resistance of probiotics, such as *L. acidophilus*, *L. paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, *L. casei*, etc. (Zhou *et al.*, 2005; Coppola *et al*., 2005; Rojo *et al.*, 2006; Maria, 2007; Belletti *et al*., 2009; Sigrid *et al.*, 2010; Roberta *et al.*, 2010; Sigrid, 2010). It was showed that many strains were resistant to most of antibiotics, and their antibiotic tolerances were variable, species-dependent and related to the product types. If the antibiotic resistance could not be transferred, the worry about the safety was not necessary. Zhou *et al.* (2005) reported the susceptibility of new probiotic *Lactobacillus* and *Bifidobacterium* strains to antibiotics. No significant clinically transmissible antibiotic resistance genes were found among the tested probiotic strains, including *L. rhamnosus* HN001 and HN067, *L. acidophilus* HN017 and B. lactis HN019. However, Pier *et al.* (2003) indicated the presence of plasmid pCF10 that encodes tetracycline resistance. This plasmid pCF10 could be transferred from *Enterococcus faecalis* OG1rf cells to food strain *E. faecalis* BF3098c with high frequency during cheese and sausage fermentation. The transferability of resistance-related genes is unsafe.

Studies have shown that genes associated with antibiotic resistance are located in plasmids and transposons (Doucet *et al*., 1992; Mayya *et al*., 2011). Both plasmids and transposons, as important genetic substances, provide the possibility of transferability for resistance genes between bacteria. Miriam *et al*. (2010) proved the transferability of tetracycline resistance in *E. italicus* LMG 22195 from fermented milk. Strain LMG 22195 was found to contain a tetS gene located on a plasmid of approximately 20 kb. Filter mating demonstrated that the tetS gene was transferable from LMG 22195 to the recipient E. faecalis JH2-2. PCR and southern blot experiment proved that *E. faecalis* JH2-2 acquired the tetS-carrying plasmid by conjugation. A similar study also indicated that the erythromycin resistant plasmid (pAMβ1) was transferred from *Lactococcus lactis* SH4174 to *L. lactis* Bu2-60 (Joanna *et al*., 2008) and from *Lactococcus lactis* to *Enterococcus faecalis* of BALB/c mice intestinal bacteria (Igimi *et al*., 1996). Moreover, the 10877 bp tetracycline resistance plasmid pMD5057 from *L. plantarum* 5057 was completely sequenced (Morten, 2002).

So, the security issues about beneficial bacteria presently are focused on antibiotic resistance caused by the transferability of resistance genes. The lactic acid bacteria carrying resistance genes, if going into the body and food, will be unsafe. Therefore, assessment of the antibiotic-resistant genes and their transferability of potentially probiotic lactic acid bacteria used in food industry are necessary.

Conclusions

With antibiotic susceptibility discs, the susceptibility of *L. pentosus* P and Ind-3 from fermented products to streptomycin and kanamycin was surveyed. The results showed that the two trains were resistant to kanamycin. The strain of Ind-3 was resistant to streptomycin, but the strain of P was sensitive. The genes responsible for resistances to kanamycin [aph(3")-III] were found in plasmid of *L. pentosus* P and Ind-3 by PCR. The resistant plasmid was highly similar to *E. faecalis* plasmid pSL1. The transferability of the resistant plasmid is under study.

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